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Non-neurogenic SVZ-like niche in dolphins, mammals devoid of olfaction

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Abbreviated title: Absence of adult neurogenesis in dolphins

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30 **Abstract**

31 Adult neurogenesis has been implicated in brain plasticity and brain repair. In mammals, it is
32 mostly restricted to specific brain regions and specific physiological functions. The function and
33 evolutionary history of mammalian adult neurogenesis has been elusive so far. The largest
34 neurogenic site in mammals (subventricular zone, SVZ) generates neurons destined to populate the
35 olfactory bulb. The SVZ neurogenic activity appears to be related to the dependence of the species
36 on olfaction since it occurs at high rates throughout life in animals strongly dependent on this
37 function for their survival. Indeed, it dramatically decreases in humans, who do not depend so much
38 on it. This study investigates whether the SVZ neurogenic site exists in mammals devoid of
39 olfaction and olfactory brain structures, such as dolphins. Our results demonstrate that a small SVZ-
40 like region persists in these aquatic mammals. However, this region seems to have lost its
41 neurogenic capabilities since neonatal stages. In addition, instead of the typical newly generated
42 neuroblasts, some mature neurons were observed in the dolphin SVZ. Since cetaceans evolved from
43 terrestrial ancestors, non-neurogenic SVZ may indicate extinction of adult neurogenesis in the
44 absence of olfactory function, with the retention of an SVZ-like anatomical region either vestigial
45 or of still unknown role.

46

47

48 **Introduction**

49 Adult neurogenesis is a widely-conserved feature in vertebrates, generally undergoing
50 ‘phylogenetic reduction’ from amphibians to humans within tetrapods (Kempermann, 2012;
51 Grandel and Brand, 2013). Despite remarkable discoveries leading to a better understanding of this
52 process, the underlying logic of adult neurogenesis in evolution, as well as its function, are still a
53 matter of debate. In all mammals studied so far, lifelong neurogenesis persists within two canonical
54 neurogenic sites (Feliciano et al., 2015) or stem cell niches: the subventricular zone located in the
55 forebrain (SVZ; Tong and Alvarez-Buylla, 2014) and the subgranular zone of the dentate gyrus in
56 the hippocampus (SGZ; Vadoaria and Gage, 2014). The production of new neurons acts as a sort of
57 ‘metaplasticity’ (second-level plasticity) primarily linked to learning tasks performed within specific
58 neural systems, such as olfactory learning within the olfactory bulb (Lepousez et al., 2013;
59 Sakamoto et al., 2014) in addition to memory and pattern separation in the hippocampus (Aimone et
60 al., 2014; Sahay et al., 2011). However, the ultimate function/aim of adult neurogenesis as a
61 conserved biological process is far from being identified. Substantial differences exist in the
62 extension and importance of neurogenic sites with respect to species, age, brain region and
63 ecological niche, thus making it difficult to identify any common traits (Barker et al., 2011;
64 Bonfanti and Peretto, 2011; Sanai et al., 2011; Amrein, 2015; Kempermann, 2016).

65 In terrestrial mammals, the SVZ is the largest neurogenic site (Bordiuk et al., 2014) which provides
66 new neurons for the olfactory bulb through the rostral migratory stream (Lois and Alvarez-Buylla,
67 1994). The SVZ neurogenic activity appears related to the importance of olfaction, since it occurs at
68 high rates throughout life in animals strongly dependent on olfactory functions for their survival
69 (e.g., rodents; Lepousez et al., 2013). Whereas in humans, who have smaller olfactory bulbs and do
70 not depend so much on olfaction the production of new neurons dramatically decreases with age
71 (Sanai et al., 2011). Apart from the lack of a deeper understanding of this trend, it is still unknown
72 whether the existence of adult SVZ neurogenesis is actually dependent on olfactory functions and

73 related brain structures. Additionally, the search for the answer to the pivotal question of the
74 evolutionary interpretation of the functions of neurogenesis during the tetrapod evolution still
75 remains unanswered. Hence, in this study we investigated whether the forebrain neurogenic niche is
76 present in natural animal models devoid of olfaction, namely, the dolphins. Marine Cetartiodactyla
77 live underwater and have developed alternative techniques for navigation, foraging and tracking of
78 prey (echolocation; Marriott et al., 2013). Thus, unlike terrestrial mammals and fish, they possess
79 significantly reduced or absent olfactory systems (Breathnach, 1953; Breathnach and Goldby, 1954;
80 Oelschläger, 2008). Even within other adult cetaceans, such as mysticetes, which possess a reduced
81 olfactory system (Oelschläger and Oelschläger, 2009), dolphins have completely lost olfaction
82 (Oelschläger, 2008; Cozzi et al., 2017). The terminal nerve is the only surviving component of the
83 three functional systems, namely, the olfactory, vomeronasal, terminal systems in the nasal region
84 of the mammalian's head (Ridgway et al., 1987). A recent report (Parolisi et al., 2015),
85 demonstrated that neonatal dolphins lack the thick SVZ germinative layer typically persisting at
86 birth on the ventricle wall of terrestrial mammals (Tramontin et al., 2003; Peretto et al., 2005),
87 including humans (Del Bigio, 2011; Sanai et al., 2011). This finding might be due to either the
88 advanced developmental stage of the dolphin brain at birth (Ridgway, 1990) or the absence of
89 olfaction, with the possibility that periventricular neurogenesis could be absent in these aquatic
90 mammals since birth. In addition, a recent study showed that several cetacean species have small
91 hippocampi which do not stain for doublecortin (Patzke et al., 2015), thus indicating the possibility
92 that adult neurogenesis itself might be lacking in these animals. Nevertheless, due to their large
93 brain size and scarce availability of tissues that are fixed well (dolphins are legally protected
94 animals on the basis of ethical and environmental issues), current knowledge by no means excludes
95 the existence of postnatal neurogenesis in these animals. In the present study, the periventricular
96 region of ten dolphins belonging to two different species (*Tursiops truncatus*, bottlenose dolphin;
97 *Stenella coeruleoalba*, striped dolphin; Fig. 1 and Table 1) and ages (neonatal and adult) were

carefully analyzed using histology and immunocytochemistry in order to investigate the presence (or absence) of a neurogenic SVZ similar to terrestrial mammals.

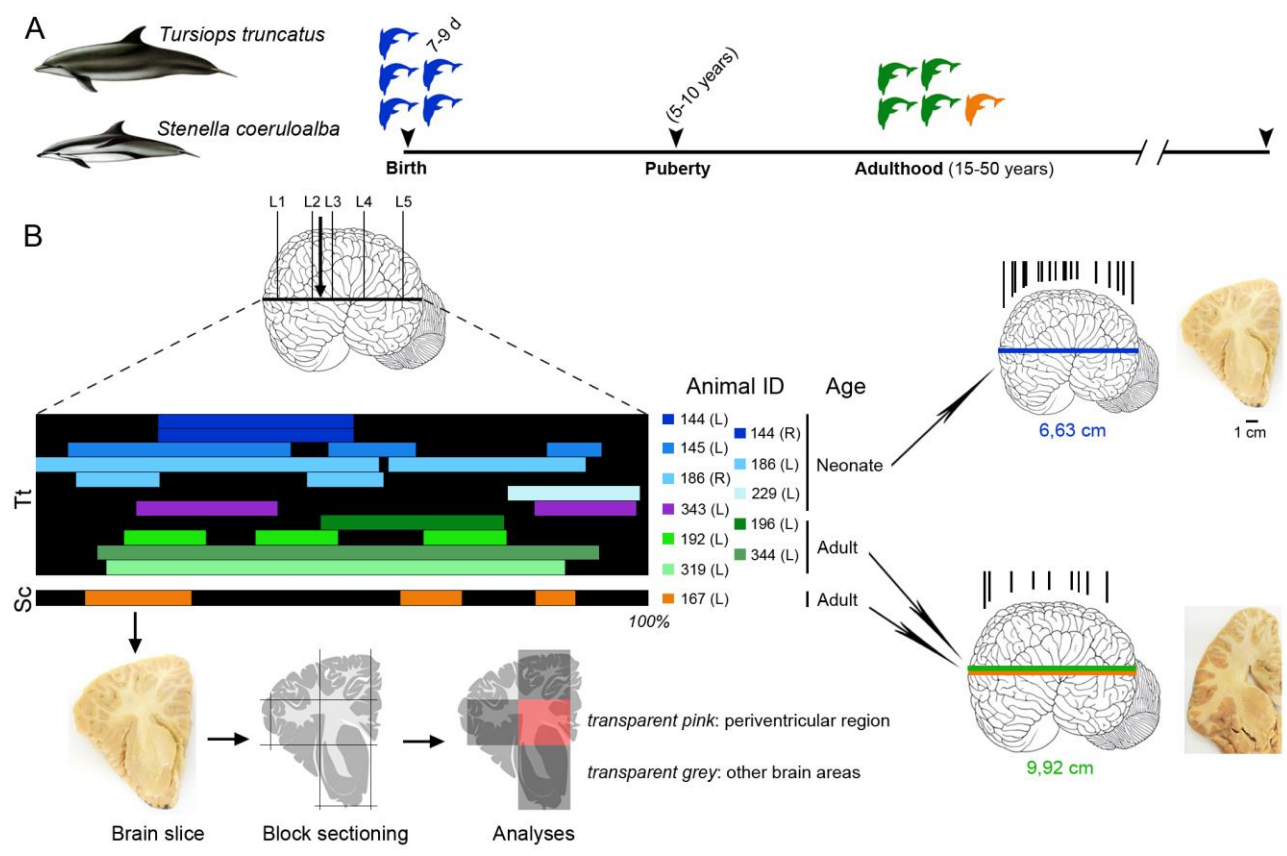


Fig 1

Materials and methods

Tissue samples

Dolphin tissues

In this study we used brain samples obtained from 10 dolphins, 9 bottlenose dolphins (*Tursiops truncatus* Montagu, 1821 - *T. truncatus*) and 1 striped dolphin (*Stenella coeruleoalba* Meyen, 1833 - *S. coeruleoalba*) stored in the Mediterranean Marine Mammal Tissue Bank (MMMTB) of the University of Padova at Legnaro, Italy (see Table 1 and Fig. 1). The MMMTB is a CITES recognized (IT020) research center and tissue bank, sponsored by the Italian Ministry of the Environment and the University of Padova, with the aim of harvesting tissues from wild and captive

112 cetaceans and distributing them to qualified research centers worldwide. The bottlenose and the
113 striped dolphins have a very similar shape and anatomy. Although, differences in size and weight
114 are evident in oceanic animals, (*T. truncatus* is generally larger than *S. coeruleoalba*) they are
115 reduced in dolphins that live in relatively smaller basins (including the Mediterranean Sea).
116 Tissue samples consisted of brain coronal slices (see Parolisi et al., 2015, Morgane et al., 1980, and
117 Fig. 1) approximately 1-1,5 cm thick, collected during post-mortem procedures performed in the
118 necropsy room of the Department of Comparative Biomedicine and Food Science of the University
119 of Padova at Legnaro, and fixed by immersion in 4% buffered formalin. Post-mortem delay before
120 actual sampling varied between a minimum of 18 to a maximum of 40 hours.
121 To confirm the immunodetection of Ki-67 antigen within an active germinal layer and to quantify
122 its cell proliferation density, we sampled tissue blocks from the top of the left cerebellar hemisphere
123 from neonatal dolphins to investigate the immunodetection of Ki-67 antigen within an active
124 germinal layer and to quantify its cell proliferation density (see Parolisi et al., 2015).

125

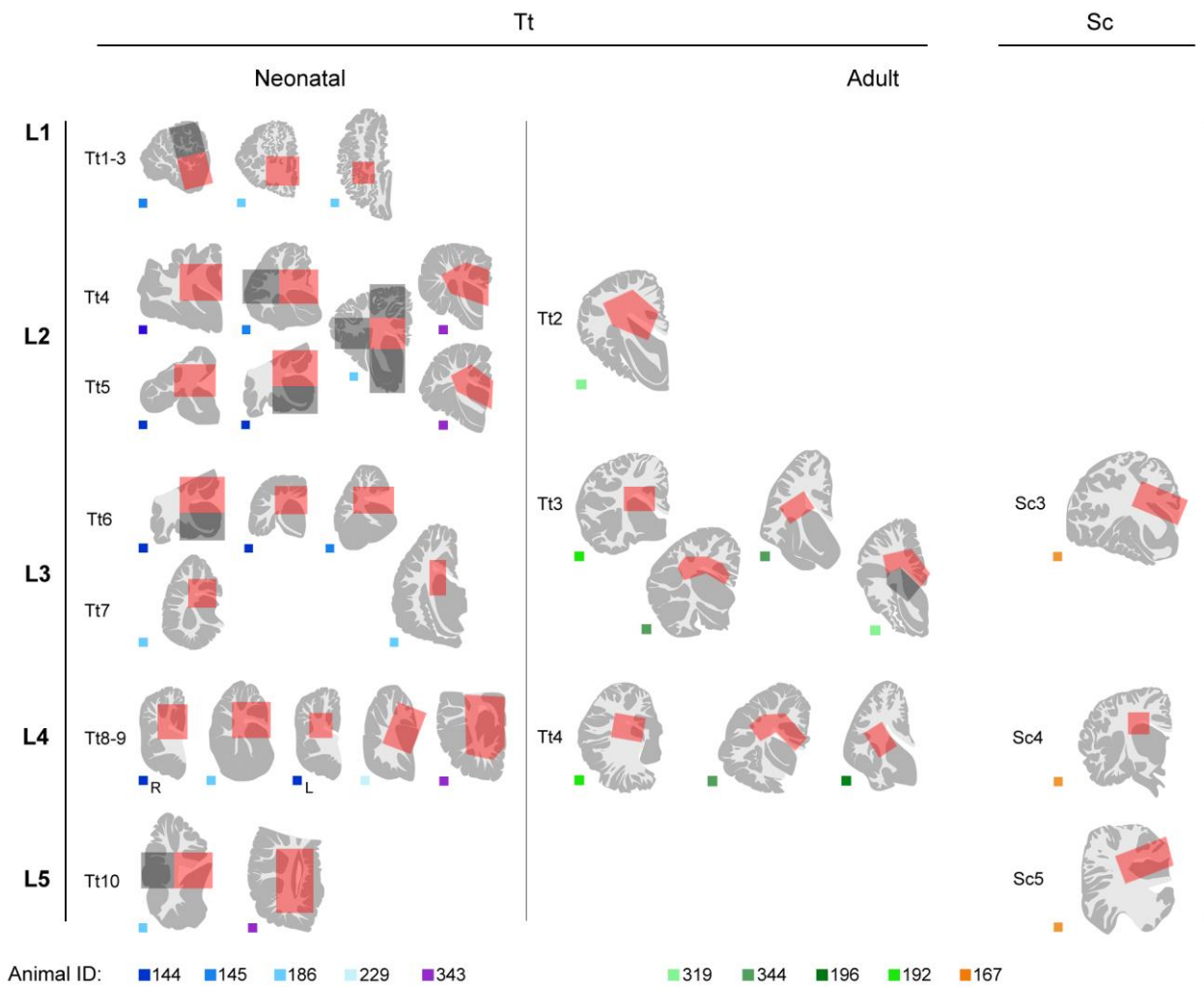
126 **Gross anatomy of the dolphin tissue slices**

127 To obtain a representation of single brain levels, the anterior face of thick brain slices was
128 photographed and imported on Neurolucida (Micro-Brightfield, Colchester, VT). Here, the outlines
129 of each coronal section, including those of the external (pial) surface and those at the white
130 matter/grey matter limits, were drawn (Fig. 1B). The contours were then imported to Photoshop to
131 obtain images of each brain level. The whole procedure has been described previously in detail
132 (Parolisi et al., 2015).

133

134 **Tissue processing for histology and immunocytochemistry**

135 Smaller blocks were cut from thick, formalin-fixed tissue slices (about 1,5x2,5 cm; see Fig. 1 and
 136 Parolisi et al., 2015), washed in 0.1M phosphate buffer (PB), pH 7.4, for 24 hours, then
 137 cryoprotected in graded concentrations of sucrose solutions up to 30% in 0.1M PB and
 138 subsequently frozen by immersion in liquid nitrogen-chilled isopentane at -80°C. Cryostat sections
 139 (40 µm thick) were cut on glass slides treated with 3-Aminopropyltriethoxysilane (Sigma-Aldrich,
 140 741442) and processed for histological and immunocytochemical analyses. All thick slices and
 141 relative blocks used in this study at different anterior-posterior brain levels and ages are
 142 summarized in Fig. 2.



144 Fig. 2

145 For immunocytochemical analysis, two different protocols of indirect staining were employed
 146 namely, the peroxidase or the immunofluorescence techniques. In peroxidase protocol, the sections

147 were pre-incubated in 1% H₂O₂ - phosphate saline buffer (PBS) for 20 min, rinsed in PBS and then
148 pre-incubated in blocking buffer (3% horse serum (HS), 2% bovine serum albumin (BSA), 1%
149 Triton X-100 in 0.01 M PBS, pH 7.4) for 1h at room temperature to reduce non-specific staining.
150 Then the sections were incubated for 24–48 h at 4°C in a solution of 0.01M PBS, pH 7.4,
151 containing 0.5% Triton X-100, 2% HS, 1% BSA and the primary antibody. Immunohistochemical
152 reactions were performed by the avidin–biotin–peroxidase method (Vectastain ABC Elite kit;
153 Vector Laboratories, Burlingame, CA, USA) and revealed using 3,3'-diaminobenzidine (3% in Tris-
154 HCl) as chromogen. Sections were counterstained with Cresyl violet staining, according to standard
155 procedures described previously (see Ponti et al. 2006a,b), mounted with DPX Mountant (Sigma-
156 Aldrich, 06522) and examined using an E-800 Nikon microscope (Nikon, Melville, NY) connected
157 to a colour CCD Camera. In immunofluorescence staining the sections were rinsed in PBS and then
158 pre-incubated in blocking buffer (3% horse serum (HS), 2% bovine serum albumin (BSA), 1-2%
159 Triton X-100 in 0.01 M PBS, pH 7.4), for 1h at room temperature. Then the sections were incubated
160 for 24–48 h at 4°C in a solution of 0.01M PBS, pH 7.4, containing 1-0.5% Triton X-100, 2% serum,
161 1% BSA and the primary antibody. Following primary antisera incubation, sections were incubated
162 with appropriate solutions of secondary cyanine 3 (Cy3)-conjugated (1:800; Jackson
163 ImmunoResearch, West Grove, PA) and Alexa 488-conjugated (1:800; Molecular Probes, Eugene,
164 OR) antibodies, for 2 hours RT. Sections were counterstained with 4',6-diamidino-2-phenylindole
165 (DAPI, KPL, Gaithersburg, Maryland USA), mounted with MOWIOL 4-88 (Calbiochem, Lajolla,
166 CA). The antibodies and the dilutions used were as follows: doublecortin (DCX), polyclonal,
167 rabbit, AbCam, 1:1000-1:1800, and polyclonal goat, Santa Cruz, 1:700; GFAP, polyclonal, rabbit,
168 Dako, 1:2000; Ki-67 antigen, polyclonal, rabbit, Novocastra, 1:600-1:1000; vimentin (VIM),
169 monoclonal, mouse (40EC), Exbio, 1:800; calretinin (CR), polyclonal, rabbit, Santa Cruz, 1:200,
170 and MAP2, monoclonal, mouse, Millipore, 1:1000 (a list of antibodies tested in this study that
171 failed to demonstrate immunostating on the dolphin tissues in the present study is provided in Table
172 2). To reveal the immunohistochemical and immunofluorescence reactions, the sections were

173 examined using an E-800 Nikon microscope (Nikon, Melville, NY) connected to a colour CCD
174 Camera, a Leica TCS SP5 (Leica Microsystems, Wetzlar, Germany) confocal microscope, and a
175 Nikon Eclipse 90i. (Nikon, Melville, NY) confocal microscope.

176

177 **Image processing and data analysis**

178 All images were processed using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA). Only
179 general adjustments to color, contrast, and brightness were made. Quantitative evaluations were
180 performed through the Neurolucida software (MicroBrightfield, Colchester, VT). The parameters
181 considered were as follows: Ki-67+ cell density in SVZ-lr, ScWM, and corpus callosum (18
182 sections for neonates, 7 sections for subadult, 60 sections for adults), and in EGL (20 sections/ages);
183 distance between lateral ventricle wall and SVZ-lr (three measures performed on 33 sections for
184 neonates and 12 sections for adults); evaluation of the continuous gap between SVZ-lr and ScWM
185 cell clusters (31 sections in neonates, at L3). The averages measured of the cell body diameter of
186 SVZ-lr tightly-packed cells (20 cells) and SVZ-lr neurons (20 cells), diameter of ScWM cell
187 clusters (181 clusters), Cm (27 measurements) and Cms areas (69 measurements) did not deviate
188 significantly from normal distribution (Shapiro Wilk test for data $n < 30$; Anderson-Darling test for
189 $n > 30$).

190 All the graphs were constructed using Graph Pad Prism (San Diego California, USA). Statistical
191 analyses were performed by Graph Pad Prism software and included unpaired (two-tailed) Student's
192 t test (comparing only two groups). $p < 0.05$ was considered as statistically significant. Data are
193 expressed as averages \pm standard deviation (SD).

194

195

196 **Results**

197 Considering the remarkable size of the adult dolphin brain (about 10 cm length and 1,3-1,7 Kg
198 weight, in adult *T. truncatus*), and the understanding that SVZ neurogenesis is most prominent at
199 birth in terrestrial mammals, we began the analyses on neonates, using the atlas of the
200 neonatal/early postnatal dolphin forebrain as an anatomical reference (Parolisi et al., 2015). Careful
201 histological screening and immunocytochemical analyses were carried out on the entire
202 periventricular region (Fig. 2 and Table 3) in search for signs of any remnants resembling a typical
203 neurogenic niche. Staining specificity for the cytoskeletal protein doublecortin (DCX; consistently
204 expressed in newly generated neuroblasts and immature neurons; Nacher et al., 2001; Brown et al.,
205 2003) and the marker for cell proliferation, namely the Ki-67 antigen (Kee et al., 2002) was
206 confirmed by immunocytochemical detection of granule cell precursors in the external germinal
207 layer (EGL) of the neonatal dolphin cerebellar cortex, which served as internal control (Fig. 3A).
208 DCX staining was further tested in neonatal and adult dolphin brains by detection of a population of
209 immature neurons occurring in the superficial layers of the cerebral cortex of most mammals
210 studied so far (references in Bonfanti and Nacher, 2012; Fig. 3B).

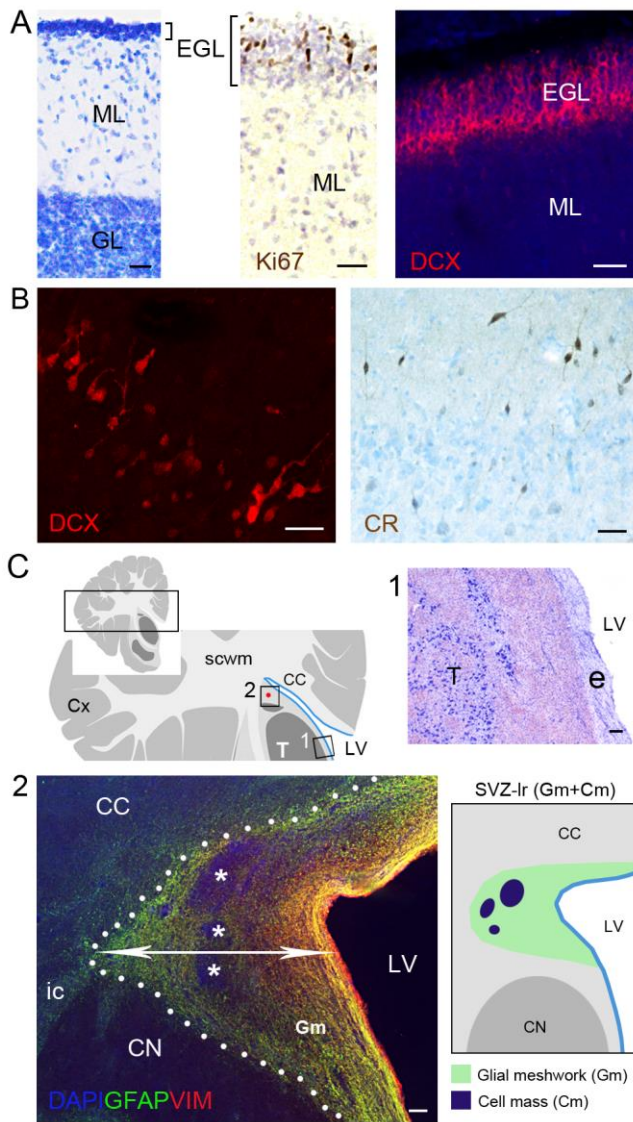


Fig 3

Identification of a SVZ-like region in neonatal and adult dolphins

Histological screening in the brain periventricular region of neonatal *T. truncatus* confirmed the absence of a well recognizable sub-ependymal germinal layer along most of the lateral ventricle wall (Parolisi et al., 2015; Fig. 3C1), yet clusters of small tightly-packed cells (3,4-0,63 μm - cell body diameter) were detected in a restricted region located at its dorsolateral corner, from level Tt3 to Tt10 (Figs. 3C2 and 4). Systematic analysis carried out on serial sections (see Table 3 for anteroposterior brain level steps) revealed that these clusters form a very thin, continuous cell mass

220 (Cm; $145615,06 \pm 68402,16 \mu\text{m}^2$ - average area at L2-L4 levels; see Table 4) lining the entire lateral
221 ventricle extension and reaching a length of approximately 4,9 cm (estimated by considering
222 consecutive brain sections cut following the beak-fluke axis that contained the Cm; see Morgane et
223 al., 1980 and Fig. 4). Immunocytochemical detection of the astrocyte marker glial fibrillary acidic
224 protein (GFAP) revealed a dense glial meshwork (Gm) completely surrounding the Cm, sharply
225 ending at the limit with the corpus callosum (dorsal and lateral) and the forebrain caudate nucleus
226 (lateral and ventral; Figs. 3 and 4). The Cm was never observed to be directly in contact with the
227 ventricular wall, maintaining an evident distance from the ependyma (Fig. 4 and below). At the
228 most anteroposterior brain levels, the Cm was split in smaller cell clusters (Cms; $6210,76 \pm 3866,14$
229 μm^2 - average area at L1 and L5 levels), their number varying from 1 to 12, being higher at the
230 extremities and lower in the middle (Fig. 4 and Table 4). The neuroblast-like nature of these cells
231 was suggested by their DCX expression (Fig. 4A). On the whole, this region appears to be
232 organized into two main cellular compartments (Cm and Gm) that seem phylogenetically related to
233 the adult SVZ described in most terrestrial mammals (Lois et al., 1996; Peretto et al., 1997,2005;
234 Bonfanti and Peretto, 2011), and thus referred hereafter to as SVZ-like region (SVZ-lr).

241

242

243 **Periventricular neurogenic processes in dolphins are almost exhausted at birth and absent in**
244 **adulthood**

245 Immunocytochemical detection of Ki-67 antigen revealed only a few scattered proliferating cells in
246 the whole SVZ-lr of neonatal animals (Fig. 5A) and none in adults. In the neonatal SVZ-lr the Ki-
247 67+ nuclei appeared evenly distributed both in Cm and Gm, their frequent appearance in doublets
248 indicative of the absence of cell migration. Quantitative analysis revealed very low rates of cell
249 proliferation (average cell density/mm² 43,16±32,92; Table 4) substantially similar to those in the
250 surrounding parenchymal tissue (Fig. 5B). In the young *T. truncatus* (subadult), such rate was even
251 lower than in the corpus callosum (Fig. 5B and Table 4) wherein a low, protracted proliferation of
252 glial cell precursors is known to occur (Dawson et al., 2003). The number of proliferating cells in
253 the neonatal dolphin SVZ-lr was found to be negligible when compared with those typically found
254 in the correspondent neurogenic site of terrestrial mammals (average cell density/mm² 2657±86, see
255 Armentano et al., 2011 and Fig. 5B, right). This is possibly indicative of the precocious exhaustion
256 of neurogenic activity at birth.

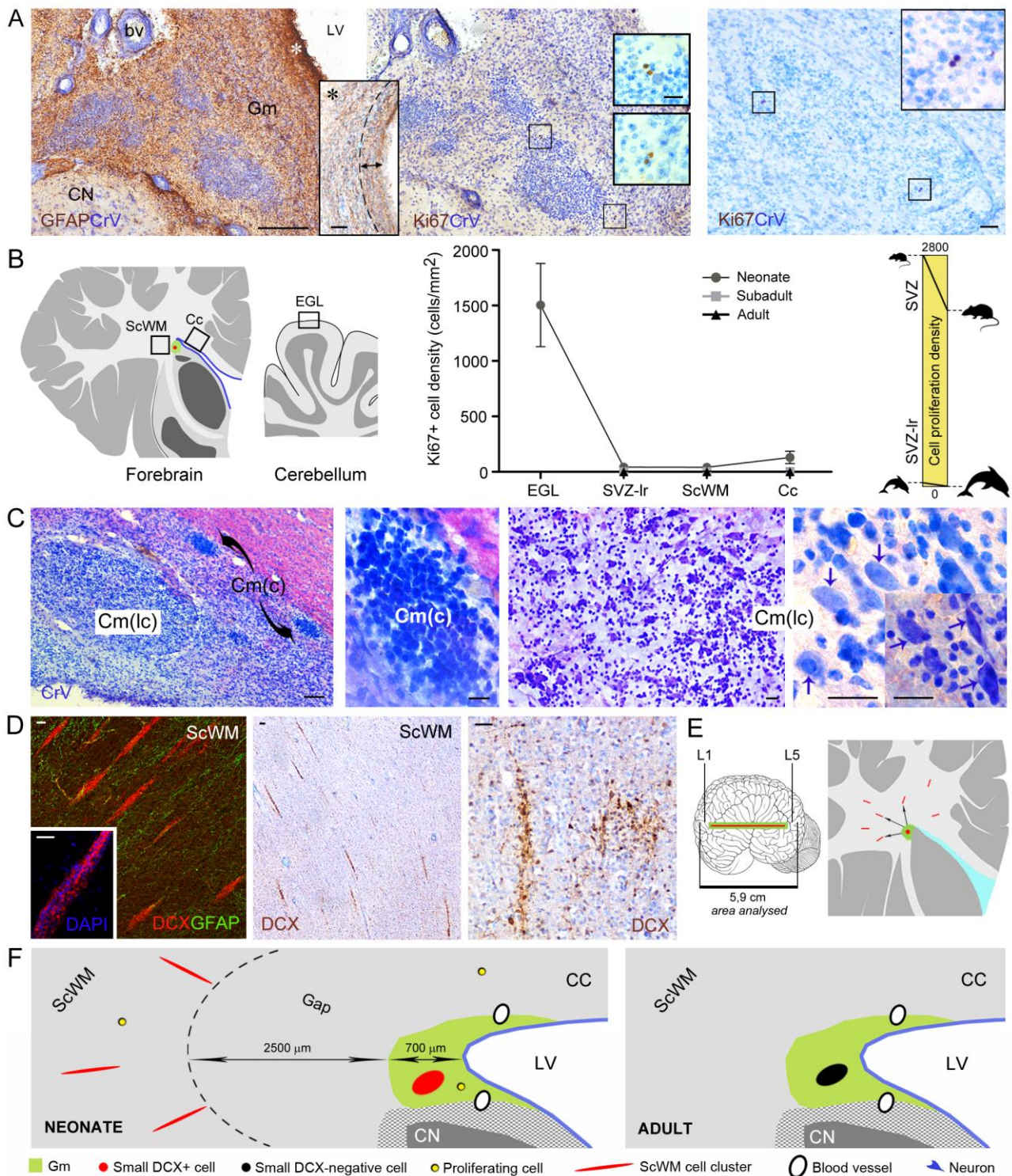


Fig 5

Other features previously unnoticed in terrestrial mammals were also observed in the internal organization of the Cms. Some of the cell clusters appeared "less compact", with small-sized cells more sparse and distant to each other (Fig. 5C). In the neonatal SVZ-lr, many of these cells were not expressing DCX (Fig. 6A) and were intermingled with larger cells morphologically

263 recognizable as neurons with triangular- or bipolar-shaped soma ($5,74 \pm 1,36 \mu\text{m}$ - cell body
264 diameter; Fig. 5C). Most of these cells were immunoreactive for the neuronal marker microtubule-
265 associated protein 2 (MAP2; Herzog and Weber, 1978; Fig. 6), whereas, a smaller population
266 (around 15-30% - value estimated on 5 sections for each age performed at the L3-L4 levels)
267 expressed the calcium-binding protein calretinin (CR; von Bohlen and Halbach, 2011; Fig. 6; see
268 Fig. 3B for internal control). The partial lack of DCX staining, along with the expression of
269 neuronal maturation markers, strongly confirm a progressive loss of neurogenic activity in the
270 dolphin SVZ-lr starting at very early ages. Similarly, no DCX staining was detectable in the SVZ-lr
271 of adult dolphins (Fig. 4B and 6A), wherein the CR⁺ neurons showed further signs of
272 differentiation, i.e. the extension of neuritic processes (Fig. 6).

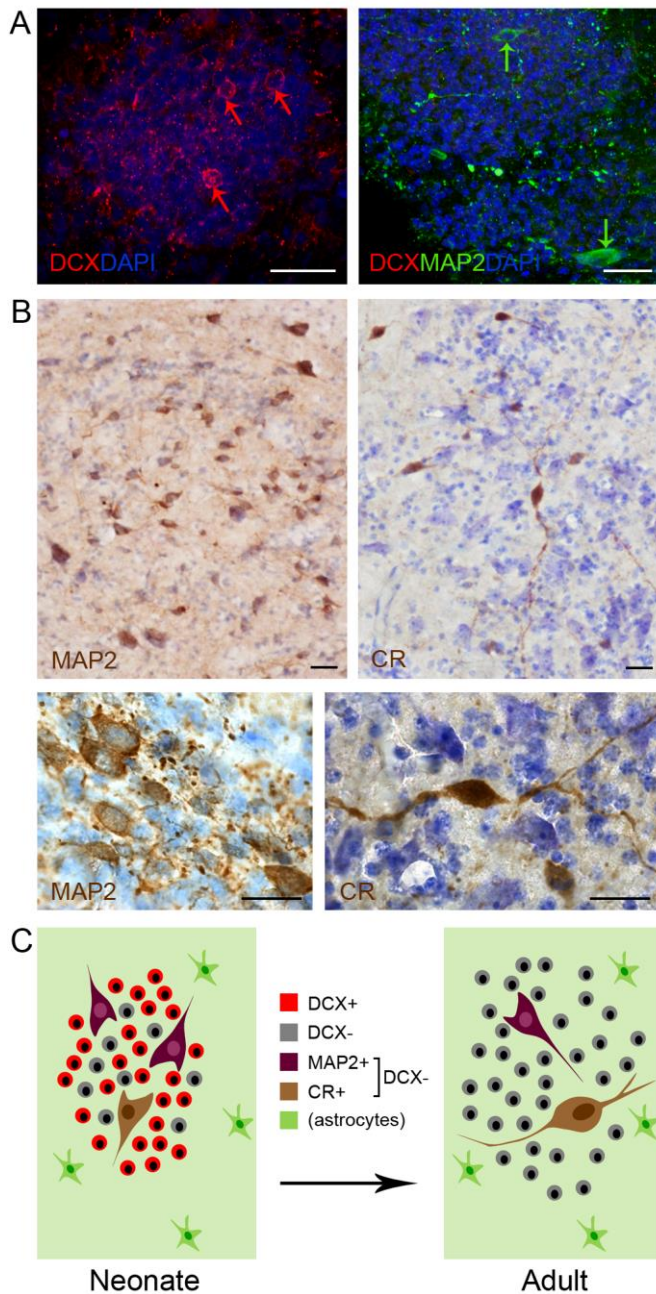


Fig 6

SVZ neurogenesis provides neuronal precursors for the olfactory bulb in all terrestrial mammals (Lois and Alvarez-Buylla 1994; Bonfanti and Ponti, 2008). Thus, the occurrence of an SVZ-lr in the brains of aquatic mammals raises the question as to whether some streams do exist in spite of the absence of olfaction/olfactory bulb in these animals. To answer this question, the subcortical white matter (ScWM) surrounding the entire SVZ-lr was analyzed at all ages in search for DCX+ cells/streams. In the neonates, elongated clusters of small, tightly-packed cells were detectable (Fig. 5D). Both compact, thick and less compact, thin clusters ($37,52 \pm 35,47 \mu\text{m}$ - transverse diameter,

with substantial variability in different animals) were observed (Fig. 5D). They were mostly radially-oriented in large portions of the ScWM, occupying a fan-shaped area along the inner part of the hemisphere (anteriorly, laterally, ventrally, and posteriorly to the SVZ-lr), yet never reaching the cortex. Since the shape of these structures might be somehow reminiscent of the "parenchymal chains" of neuroblasts previously described in other mammals (Luzzati et al., 2003; Ponti et al., 2006a), they were investigated for possible presence of dividing cells and/or connection with the SVZ-lr and its Cms. No Ki-67+ cells were ever detectable in association with the ScWM cell clusters, although a few proliferating cells were occasionally found in the tissue among the clusters (Fig. 5F). Upon careful analysis carried out all along the SVZ-lr (see Fig. 5E and Table 3 for serial section steps), it was found that no direct connections ever occurred between the SVZ-lr and any of the ScWM cell clusters. Rather, a continuous "gap" completely devoid of cell clusters (2500 ± 200 μ m thick; Fig. 5E,F) was present in the areas surrounding the SVZ-lr, in every direction, including anterior and posterior aspects, thus excluding the possibility that they are continuous streams of cells generated within the SVZ-lr.

Discussion

The brains of all terrestrial mammals host a remnant of the periventricular, embryonic germinal layer (SVZ) particularly prominent at birth (Tramontin et al., 2003; Peretto et al., 2005) and persisting throughout life as a major neurogenic site (Kriegstein and Alvarez-Buylla, 2009; Bordiuk et al., 2014). Here we show that dolphins, although lacking such a layer, host a very small SVZ-lr located at a remote tip of the lateral ventricle, which can be consistently found at neonatal and adult ages. The SVZ-lr occupies an area approximately similar the real size of its counterpart in mice

(Fig. 4C), whose brain, in comparison, is 40 fold smaller if a correspondent coronal section area is measured, and 3000 fold smaller if the weight or volume are considered (Rose et al., 2006; Marino et al., 2000).

Unlike the SVZ of terrestrial mammals (Tramontin et al., 2003; Lois et al., 1996; Peretto et al., 1997,2005), the SVZ-lr of dolphins is already compartmentalized soon after birth with its structure being reminiscent of adult neurogenic sites (Ponti et al., 2006a; Bonfanti and Ponti, 2008). This phenomenon, although unusual in mammals, fits well with the highly advanced developmental stage of the brain in neonatal aquatic mammals (Parolisi et al., 2015), which is related to the immediate need of the newborn to already possess all the swimming competences required for life, including the ability to reach the surface and breathe (Ridgway, 1990). Yet, it is surprising that a brain region sharing features (location, inner histological organization and some molecular aspects) with the SVZ neurogenic niche of terrestrial mammals does persist in dolphins, apparently in contrast with the absence of olfaction/olfactory structures. What appears to be unique in this SVZ-lr is its extremely low rate of cell proliferation detectable in neonates, followed by utter disappearance. The density of dividing cells revealed by Ki-67 antigen localization in the SVZ-lr of the neonatal dolphins ($43,16 \pm 32,92$) is 34 fold lower when compared with the germinal layer of the cerebellar cortex in the same animals ($1504,63 \pm 374$; Figs. 3 and 5 and Table 4), 62 fold lower than that existing in the SVZ of neonatal rodents (2657 ± 86 ; Armentano et al., 2011), 47 fold lower than in adult rodents ($2018,5 \pm 420$; Rolando et al., 2012; Fig. 5), and it is not higher than in the surrounding brain parenchyma (Fig. 5B). Even in humans, despite a dramatic reduction of SVZ thickness with age (Sanai et al., 2011), a highly proliferative region exists in neonates, which persists to a lesser extent in adult and old individuals (Eriksson et al., 1998; Sanai et al., 2004; Wang et al., 2011).

The very early exhaustion of peri-ventricular neurogenic activity in dolphins is also reflected by the cellular and molecular features of the Cms in the SVZ-lr. In neonates, the small, neuroblast-like

cells are not tightly-packed, show variable and incomplete DCX staining and are intermingled with neurons expressing mature neuronal markers such as MAP2 and CR (Figs. 5-7).

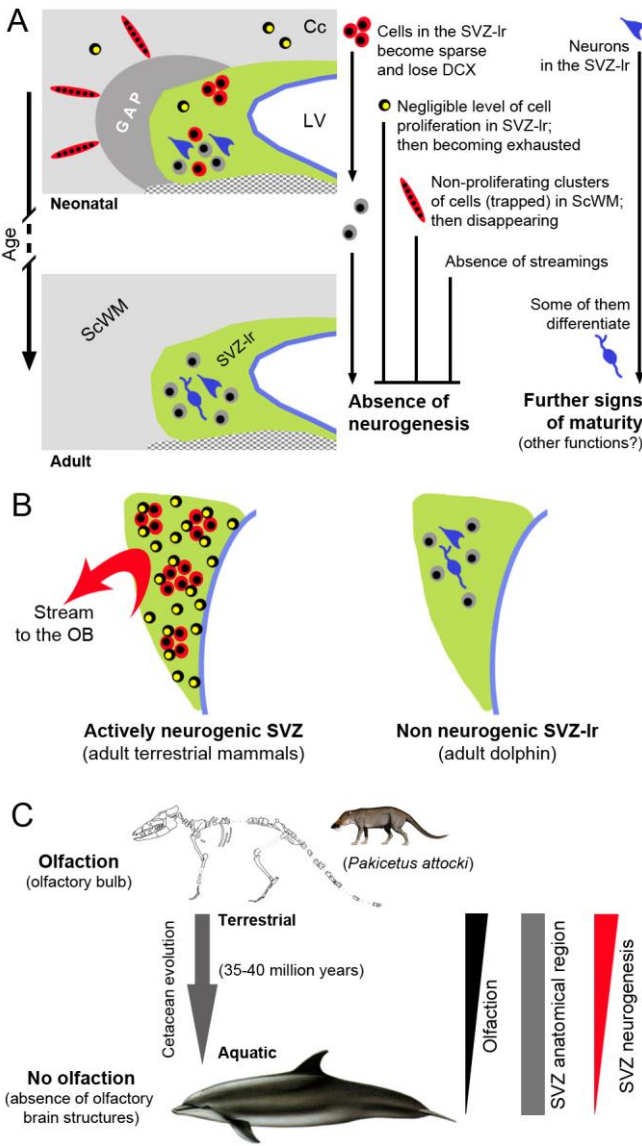


Fig 7

In adults, no DCX staining is detectable, whereas the SVZ-Ir neurons are still detectable, a subpopulation of them showing further traits of differentiation such as the extension of neuritic processes (Fig. 6). Hence, in the dolphin SVZ-Ir an early exhaustion of cell division goes in parallel with neuronal maturation. Such differentiation "in situ" might simply be a consequence of the cellular/molecular environment of the SVZ-Ir (e.g., absence of any continuous supply of new, young neuroblasts) which is no more supportive as an active stem cell niche. These observations are in sharp contrast with the current knowledge on the SVZ of all terrestrial mammals, characterized

343 by an embryonic-like tissue which persists into adulthood (Fig. 7), although with different degrees
 344 of proliferative activity from rodents to humans (Ponti et al., 2013; Eriksson et al., 1998; Wang et
 345 al., 2011; Sanai et al., 2004,2011). Additionally, a careful analysis extended to the brain regions
 346 surrounding the SVZ-lr did not reveal any streams of cells spanning from the periventricular Cms to
 347 any other direction, unlike terrestrial mammals which all exhibit a marked rostral migratory stream
 348 at perinatal stages (Lois and Alvarez-Buylla 1994; Peretto et al., 2005). Although the radially-
 349 oriented, DCX+ cell clusters present in the ScWM of neonates were reminiscent of "chain-like"
 350 structures (Luzzati et al., 2003; Ponti et al., 2006a), the occurrence of a continuous white matter gap
 351 (absence of any direct contact with the SVZ-lr perimeter) along with the scarcity of cell divisions in
 352 the SVZ-lr itself, exclude the possibility that they can represent any product of an ongoing
 353 neurogenic activity.

354 Once it is established that in dolphins all SVZ neurogenic processes are substantially exhausted at
 355 birth, clusters of DCX+ cells still present in the SVZ-lr and white matter of neonatal animals are a
 356 matter of further investigation. The occurrence of DCX+ cells in the periventricular white matter or
 357 in the corpus callosum has been previously shown in, large-brained mammals at postnatal ages
 358 (Fung et al., 2011). Although DCX is commonly expressed in newly generated neuroblasts (Brown
 359 et al., 2003), staining for this cytoskeletal protein alone is not at all a proof for the occurrence of
 360 neurogenesis, since DCX is heavily present in non-newly generated adult cell populations (Gomez-
 361 Climent et al., 2008; Luzzati et al., 2009; Bonfanti and Nacher, 2012). Considering the extremely
 362 rapid developmental growth of the dolphin brain and its remarkably advanced stage of maturation at
 363 birth (Ridgway, 1990; Parolisi et al., 2015) the ScWM cell clusters appear to be previously
 364 migrating streams of cells "trapped" in the thick white matter which fills the central part of the
 365 hemispheres, as sort of "remnants" of the last neurogenic wave. In fact, no more DCX+ cells are
 366 detectable in the entire ScWM of adults.

367 In this study, morphological, antigenic, proliferative aspects converge to support the conclusion that
 368 the dolphin SVZ-lr is a vestigial structure not behaving as an active neurogenic site since very early

369 postnatal stages (Fig. 7). This finding is consistent with previous studies that demonstrate that
370 several cetacean species have small hippocampi which do not stain for DCX (Patzke et al., 2015),
371 and strongly indicate that adult neurogenesis is totally lacking in dolphins. The two main findings
372 of this study that can have evolutionary considerations are: 1) the lack of clear signs of active
373 neurogenesis in aquatic mammals devoid of working olfaction/olfactory brain structures, and 2) the
374 counterintuitive existence of an SVZ-like region throughout their lifespan. The former observation
375 supports the occurrence of a strict relationship between adult SVZ neurogenesis and olfaction,
376 confirming the hypothesis that in mammals the production of highly specific populations of new
377 neurons is selectively destined for physiological roles such as learning, memory and plasticity
378 (Bonfanti, 2011; Peretto and Bonfanti, 2014; Obernier et al., 2014). On the other hand, the
379 persistence of an anatomical region reminiscent of the SVZ neurogenic niche (in fact, non-
380 neurogenic at all) plays against the simple hypothesis that a mammal lacking olfaction should not
381 possess an SVZ-like region. The explanation for this might be found in the evolutionary history of
382 these animals. Dolphins, and more in general cetaceans, evolved from terrestrial artiodactyls that
383 returned to the sea 35-40 million years ago (Thewissen et al., 2001). Data from fossil studies show
384 that the terrestrial ancestors of dolphins were wolf-sized terrestrial carnivorous (*Pakicetus*)
385 endowed with olfactory structures (Gingerich et al., 1983; Kishida et al., 2015). Then, in the early
386 Eocene period, by undergoing a gradual and branched transition from land to sea, they lost the
387 capacity to perceive odors (Gingerich et al., 1983; Thewissen et al., 2001). Although dolphin
388 fetuses possess small olfactory structures, they regress completely shortly after birth (Buhl and
389 Oelschläger, 1988; Cozzi et al., 2017). Adult dolphins only possess the terminal nerve, originating
390 from the olfactory placode and reaching the basal telencephalon (Buhl and Oelschläger, 1988).
391 While in adult terrestrial mammals, including man, the terminal system is reduced to a few hundred
392 neurons, in adult bottlenose dolphins (and other delphinid species), fiber strands and interspersed
393 ganglia enter the olfactory tubercle and the pre-piriform cortex (Ridgway et al., 1987). Yet, apart
394 from its common developmental origin with the olfactory system, the terminal nerve system is

395 completely independent from the SVZ germinal layer, both anatomically and functionally (Buhl and
396 Oelschläger, 1986).

397 Therefore, the retention of the SVZ-lr in extant dolphins as an anatomical region having lost any
398 neurogenic capacity strongly suggests a slow extinction of adult neurogenesis in mammals not
399 dependent on olfaction for survival. The findings of the present study also open up the possibility
400 that non-neurogenic SVZ could have changed its role over time, from neurogenesis to new, yet
401 unknown roles. However, the latter aspect would hardly be an object of investigation in ethically
402 protected animals such as the toothed whales, unless new methods of analysis are developed in the
403 future.

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420 **References**

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632 **Figure legends**

633

634 **Figure 1.** Animals and brain tissue samples used in this study (see also Table 1 and Parolisi et al.,
635 2015). A, Ten specimens belonging to two species of dolphins (*T. truncatus*, Tt; *S. coeruleoalba*,
636 Sc) at different ages (same colours as in B) were used. B, Arrow, coronal cutting direction to obtain
637 thick brain slices (examples on the right). ID, identification numbers; L, left hemisphere; R, right
638 hemisphere. Coloured lines indicate the amount of tissue available for
639 histological/immunohistochemical analyses in each animal and hemisphere (neonatal Tt, shades of
640 blue; adult Tt, shades of green; adult Sc, yellow), as a percentage of the whole brain extension
641 (black backclot; not in scale).

642

643 **Figure 2.** Tissue blocks analysed at different brain levels in all animals and ages (colors explained
644 in Fig. 1B).

645

646 **Figure 3.** Identification of an SVZ-like region (SVZ-lr) in the neonatal dolphin brain (*T. truncatus*)
647 and internal controls based on cell populations typically identified by DCX, CR, and Ki-67 antigen
648 in cerebral and cerebellar cortices of the same animals. A, Actively proliferating granule cell
649 precursors in the external germinal layer (EGL) of neonatal, as an internal control for Ki-67 antigen;
650 GL, granule cell layer; ML, molecular layer. B, Cortical neurons as an internal control for DCX (see
651 Bonfanti and Nacher, 2012) and calretinin (CR). C, No signs of residual germinal layer are
652 detectable along the lateral ventricle wall (1). General features reminiscent of the terrestrial
653 mammal SVZ are recognizable in a very small region (2), comprised between caudate nucleus
654 (CN), corpus callosum (CC) and ventricular corner: cell masses composed of tightly-packed cells
655 (Cm, asterisks) are surrounded by a dense, GFAP+, Vim+ astrocytic glial meshwork (Gm); Gm and

656 Cm form the area referred to as SVZ-lr (dotted line on the left; green area on the right). T, thalamus;
657 Cx, cortex; ic, internal capsule; scwm, subcortical white matter. Scale bars: 50 μ m.

658

659 **Figure 4.** Histological and immunocytochemical characterization of the SVZ-lr in neonatal and
660 adult dolphins (*T. truncatus* and *S. coeruleoalba*). A, Topographical position of the Cms (small red
661 dots, left), their profile (red areas, middle), and their detailed neuroanatomical location (right) at
662 different anterior-posterior brain levels. Cms are indicated by arrowheads in CrV stained sections
663 and by asterisks in immunofluorescence images; most of the small, tightly-packed cells are DCX+
664 and are surrounded by a GFAP+ astrocytic glial meshwork (Gm, green in the schematic drawings
665 on the right, illustrating the compartmentalized architecture of the SVZ-lr); CN, caudate nucleus; T,
666 thalamus; LV, lateral ventricle; bv, blood vessels. B, Same analyses carried out on brains of adult
667 animals, in both species; the profile of the Cms is black, since no DCX staining is detectable in their
668 cells. C, Left, anterior-posterior extension of the SVZ-lr in the neonatal dolphin (not showed in
669 adults since very similar); in blue, the lateral ventricle. Right, absolute and relative size of the
670 dolphin SVZ-lr and its Cms (absolute size: areas measured on 33 sections for neonates and 12
671 sections for adults; relative size: % absolute area with respect to coronal brain slice area; analysed at
672 L2) at different ages; the SVZ-lr is slightly enlarged in adults whereas the Cms are substantially
673 unchanged. Scale bars: A, 200 μ m (right bottom, 50 μ m); B, left, 1000 μ m; right, 50 μ m.

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675 **Figure 5.** Estimation of neurogenic activity in the SVZ-lr and surrounding parenchyma of neonatal
676 (A, C left, D) and adult (C, right) dolphins. A, Left, no particular density of astrocytic cells forming
677 the glial meshwork (Am) is detectable close to the ventricular wall (the darker area is an optical
678 effect due to thickness of the brain section; see inset); middle and right, a few scattered dividing
679 cells revealed by Ki-67+ nuclei are detectable in the whole SVZ-lr area of neonatal dolphins,
680 randomly distributed in the Cms, at their limits or in the Gm. B, Quantification of proliferating cell
681 density in the dolphin SVZ-lr, subcortical white matter (ScWM), and corpus callosum (Cc) at

different ages; squares indicate the areas analysed; cell division rate is very low in the SVZ-lr of neonates, substantially matching that in the parenchymal tissue (middle), being 34 fold lower than in the cerebellar external germinal layer (EGL) and 62 fold lower than in the SVZ of neonatal mice (right). No cell division is detectable in adults (graphically represented in F). C, At all ages and species, in the SVZ-lr both compact (c) and less compact (lc) cell masses (Cm) are present, the latter also harboring large cells with neuronal morphology (right, blue arrows). CN, caudate nucleus; CrV, cresil violet; bv, blood vessels. D, Clusters of tightly-packed, DCX⁺ cells in the ScWM surrounding the SVZ-lr in neonatal dolphins; their size and compactness is variable in different individuals. After serial analysis at different brain levels (E, right; see Table 3), clusters were confined within the ScWM and no one can be ever found in direct contact with the SVZ-lr (or its Cms), a gap always existing between the most inner clusters and the SVZ-lr perimeter (F, left). No DCX⁺ cell clusters are detectable in the white matter of adults (F, right). Scale bars: A left, middle, 100 μ m, right, 30 μ m, insets, 10 μ m; C left, 50 μ m, others, 10 μ m; D, 20 μ m.

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Figure 6. Cellular organization of the Cms in the dolphin SVZ-lr at different ages (A, left and B, left bottom: neonate; A, right, B, top and right bottom: adult). A, Only scattered, small round-shaped cells are DCX⁺ in a Cm of a neonatal dolphin (left), and none in the adult (right). B, Most cells with large cell bodies found in the SVZ-lr are MAP2⁺ neurons (B, left, top and bottom; see also A, right); a smaller amount of neurons is CR⁺, showing more differentiated shapes in adults, including neurite extensions (B, right, top and bottom). C, Schematic representation of the cell types in the dolphin SVZ-lr at different ages: small, round-shaped cells in the Cms show an overall loss of DCX staining shifting from young to adult ages; neurons are already present around birth, although showing less mature morphologies than in the adult (*T. truncatus*). Scale bars: 20 μ m.

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Figure 7. SVZ-lr and neurogenesis in dolphins at different ages: comparison with terrestrial mammals. A, Several features displayed by the SVZ-lr of neonatal and adult dolphins converge to

708 the conclusion that their periventricular neurogenesis is almost exhausted at birth then being absent,
709 with progressive neuronal differentiation occurring within the SVZ-lr itself. Green, glial meshwork;
710 red, DCX+ cells; grey, DCX-negative cells; yellow dots, cell proliferation; ScWM, subcortical
711 white matter. B, Striking contrast between the typical proliferative, neurogenic SVZ of terrestrial
712 mammals and the non-neurogenic SVZ-lr of dolphins. C, Evolutionary considerations and
713 hypotheses: dolphins are cetaceans devoid of olfaction which derive from terrestrial mammals
714 endowed with olfactory structures (wolf-sized *Pakicetus*, Thewissen et al., 2001; Kishida et al.,
715 2015); an SVZ-lr (intended as an anatomical region) has been retained in extant dolphins, yet losing
716 any neurogenic capacity.

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720 **Table 1.** Detail of the sampled bottlenose dolphins. C.E., Controlled environment.
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Specimen	ID	Sex	Origin	Length/Weight	Age
<i>T. truncatus</i>	186	F	C.E.	110,5 cm/19kg	(neonatal) 19 days
	145	M	C.E.	118 cm/19kg	(neonatal) 9 days
	144	M	C.E.	117 cm/22,1kg	(neonatal) 9 days
	229	M	C.E.	99 cm/19 kg	(neonatal) 7 days
	343	F	C.E.	95 cm	(neonatal) 1 day
	344	M	Stranded	195 cm/98,5 Kg	Subadult
	192	F	Stranded	240 cm/178,5 kg	Adult
	196	M	Stranded	300 cm/219 kg	Adult
	319	M	Stranded	310 cm	Adult
<i>S. coeruleoalba</i>	167	M	Stranded	198 cm/94 kg	Adult

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726 **Table 2.** Antibodies tested in this study, not working on our dolphin tissues.
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Antigen	Antibody/antiserum	Host	Diluition	Source
BLBP	poly	rabbit	1:1000	Chemicon
Calbindin	poly	rabbit	1:10000	Swant
Calbindin	mono	mouse	1:1000	Swant
CdIIb	mono	mouse	1:1000	Sigma
DCX	poly	guineapig	1:800	Millipore
GABA	poly	rabbit	1:2000	Sigma
GFAP	mono	mouse	1:100	Millipore
GST	poly	rabbit	1:500	MBL
Iba 1	poly	rabbit	1:1000	Wako
IIIBtub	poly(Tuj1)	rabbit	1:1000	Covance
IIIBtub	mono (Tuj1)	mouse	1:100	Millipore
Ki-67	mono	mouse	1:300	BD Pharmigen
Ki-67	mono	mouse	1:200	Dako
Laminin	poly	rabbit	1:800	Dako
Map5	poly	goat	1:600	Santa Cruz
Map5	Mono	mouse	1:1500	Chemicon
MBP	mono	mouse	1:100	Millipore
NeuN	mono, A60	mouse	1:200	Millipore
Neurofilament	poly	rabbit	1:800	AbCam
Ng2	mono	mouse	1:200	Chemicon
Ng2	mono	mouse	1:300	US Biological
Ng2	mono	mouse	1:200	Upstate
Ng2	mono	mouse	1:200	Sigma
Ng2	poly	rabbit	1:400	Chemicon
Olig2	poly	goat	1:400	R&D System
Olig2	poly	rabbit	1:500	Millipore
PVim	poly	ra	1:800	AbCam
Parv19	mono	mouse	1:2000	Swant
Parv19	poly	rabbit	1:3000	Sigma
Pax 2	mono	mouse	1:800	Santa Cruz
Pax6	poly	rabbit	1:800	AbCam
PDGFRa	poly	rabbit	1:1000	Santa Cruz
PDGFRa	poly	rat	1:100	BD Pharmigen
PH3	poly	rabbit	1:500	Millipore
PH3	mono	mouse	1:300	Millipore
PSA NCAM	mono	mouse	1:900/1:2000	Biocampare
RIP	mono	mouse	1:400	Chemicon
S100B	poly	rabbit	1:3000	Swant
S100B	mono	mouse	1:10000	Sigma
Sox10	poly	goat	1:800	AbCam
Sox2	poly	rabbit	1:1000	Millipore
Sox2	poly	goat	1:400	Santa Cruz
Sox9	poly	rabbit	1:1000	Millipore
Tbr1	mono	mouse	1:600	AbCam
Tbr2	poly	rabbit	1:800	Millipore
Tenascin C	poly	rabbit	1:500	AbCam

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Table 3. Step intervals (µm) between cryostat tissue sections used for different types of analysis within and outside the SVZ-lr of the dolphin brains (see Fig. 2).

Species <i>Age</i>	Brain level	SVZ-like region				ScWM and Cc	
		Cm (CrV)	Gm (GFAP)	Cm (DCX)	Cell proliferation (Ki-67)	ScWM clusters (CrV, DCX)	Cell proliferation (Ki-67)
<i>Tt</i> <i>Neonate</i>	2	400	400	400	800	600	800
	3	400	200	200	400	400	400
	4	300	200	200	400	200	400
	5	300	400	400	400	200	400
	6	320	400	400	420	200	420
	7	320	400	400	420	400	420
	8	320	400	400	420	400	420
	9	400	400	400	800	600	600
	10	400	400	400	800	600	800
<i>Tt</i> <i>Adult</i>	2	800	1800	1200	2000	1200	1200
	3	400	1800	800	2000	800	800
	4	400	1800	800	2000	1000	1000
<i>Sc</i> <i>Adult</i>	3	400	1800	800	2000	1200	1200
	4	400	1800	800	2000	800	800
	5	400	1800	1200	2000	1000	1000
Analysed sections		879	633	738	459	1194	811

Tt, *T. truncatus*; Sc, *S. coerulealba*; Cm, periventricular cell mass; Gm, glial meshwork; ScWM, subcortical white matter; Cc, corpus callosum; CrV, cresil violet; DCX, doublecortin; GFAP, glial fibrillary acidic protein.

749 **Table 4.** Cellular and molecular features of the SVZ-lr in neonatal (1 day and 7-9 days) and adult *T.*
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Species <i>age</i>	Brain level	Cell masses (Cm)				Astrocytic meshwork (Am)		Ki-67+ cells (cells/mm ²)
		Number	Total area (μm ²)	DCX	N	GFAP	Area (μm ²)	
Tt <i>Neonatal</i> (1 day)	L2	11-10	139204,69±50298,49	+	+	+	2915883,33 ±128405,96	28,17±10,30
	L3	12-4	203093,03±38014,69	+	+	+	2235382 ±270675,11	n.d.
	L5	7-3	120030,02±51137,13	+	+	+	1141864,60 ±435986,77	20,07±10,05
Tt <i>Neonatal</i> (7-9 days)	L1	6-4	53028,95±20979,75	+	-	+	n.d.	n.d.
	L2	5-3	64972,45±25643,77	+	-	+	452090,11±87860,12	n.d.
	L3	3-1	158851,38±84676,47	+	+	+	876197,75±24983,08	78,57±84,52
	L4	2-6	91068,19±13325,03	+	+	+	688922,50±142514,73	31,35±5,20
	L5	2-6	118389,6 ±27084,12	+	-	+	4705096 ±1893947	56,55±22,45
Tt <i>Adult</i>	L2	6-8	222255,90 ±2218,19	-	+	+	7145366,66 ±783523,72	No cells
	L3	4-8	217682,52 ±6467,73	-	+	+	6044218 ±778629,68	
	L4	7-5	65976,75 ±29580	-	+	+	4621066,66 ±1570835,62	

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